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Production of an antibody Fab fragment using 2A peptide in insect cells

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2	Short title: Antibody production using 2A peptide in insect cells
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1 Abstract

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3 Antibody Fab fragments consist of heavy chain (Hc) and light chain (Lc) polypeptides 4 assembled with a disulphide bond. The production of a recombinant Fab fragment 5 requires the simultaneous expression of two genes encoding both an Hc and an Lc in the 6 In the present study, we investigated the production of Fab fragments in same host cell. 7 lepidopteran insect cells using a bicistronic plasmid vector carrying the Hc and Lc genes 8 linked with a 2A self-cleaving peptide sequence from the porcine teschovirus-1. We 9 also examined the arrangement of a GSG spacer sequence and a furin cleavage site 10 sequence with the 2A sequence. Western blot analysis and enzyme-linked 11 immunosorbent assay (ELISA) of culture supernatants showed that *Trichoplusia ni* BTI-12 TN-5B1-4 (High Five) cells transfected with a plasmid in which the Hc and Lc genes 13 were joined by the 2A sequence successfully secreted Fab fragments with antigen-binding 14 activity after self-cleavage of the 2A peptide. The GSG linker enhanced 2A cleavage 15 efficiency, and the furin recognition site was useful for removal of 2A residues from the 16 Transfection with a single plasmid that contained sequences for GSG, the furin Hc. 17 cleavage site, GSG, and the 2A peptide between the Hc and Lc genes exhibited a higher 18 productivity than co-transfection with a set of plasmids separately carrying the Hc or Lc 19 These results demonstrate that bicistronic expression with the appropriate gene. 20 combination of a furin recognition site, GSG linkers, and a 2A peptide may be an effective 21 way to efficiently produce recombinant antibody molecules in insect cells.

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1 Introduction

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3 Monoclonal antibodies and antibody fragments including Fab fragments have been 4 widely used for therapeutics and diagnostics, because they bind to antigens with high 5 levels of both affinity and specificity. Currently, mammalian cells such as Chinese 6 hamster ovary (CHO) cells and NS0 cells have been generally used as host cells for the 7 stable expression of recombinant therapeutic antibodies (1, 2). Insect cells have recently 8 been recognized as an excellent platform for the production of biologically active 9 recombinant proteins including antibody molecules (3-5). Insect cells are easier and 10 more cost-effective to cultivate than mammalian cells, and they can be grown to a high 11 cell density in suspension with a serum-free medium. They can also produce significant 12 amounts of recombinant proteins through post-translational processing and modifications 13 that are similar to those performed in mammalian cells (6, 7).

Among antibody molecules, an IgG consists of two identical heavy chains (Hc) and two identical light chains (Lc), which assemble with disulphide bonds, and an Fab fragment consists of two chains comprised of an Fd fragment (Hc) and an Lc, joined by a disulphide bond. Hence, the production of an IgG or an Fab fragment requires the simultaneous expression of two genes encoding both Hc and Lc in the same cell.

Bicistronic expression vectors have been developed to co-express two genes from a single open reading frame driven by one promoter. In bicistronic expression systems, internal ribosome entry site (IRES) sequences, which are retained in the picornavirus family, have been widely used. An IRES allows the initiation of translation in a capindependent manner, i.e., ribosomes bind internally at the initiating AUG without scanning the 5' non-translated region of the transcript (8), thereby ensuring the coexpression of genes located before and after the IRES. However, the use of an IRES sequence has several limitations (9, 10). Since the size of an IRES sequence is usually
longer than 500 nucleotides, limited cloning capacity could be a problem when a large
insert and/or a large plasmid must be used along with an IRES sequence. Furthermore,
the translation efficiency of genes separated by an IRES element is not equivalent,
because the translation efficiency of a gene placed after an IRES is much lower than that
of a gene located before an IRES (11).

7 2A peptides were also identified among the picornavirus family but in different sub-8 groups such as in Aphthovirus and in equine rhinitis A virus (9). 2A peptides are 9 relatively short oligopeptides (approximately 20 amino acids) located between the viral 10 P1 and P2 proteins. The 2A peptides can undergo self-cleavage between the last two 11 amino acids by preventing the formation of a normal peptide bond between glycine and 12 the last proline in order to generate mature P1 and P2 proteins, which results in the 13 ribosome skipping to the next codon during protein translation (9, 10, 12). Similar 2A 14 sequences have been found in several other viruses; the most commonly used are from 15 the foot-and-mouth disease virus (F2A) (13), the equine rhinitis A virus (E2A), the Thosea 16 asigna virus (T2A), and the porcine teschovirus-1 (P2A). Reportedly, P2A has a higher 17 cleavage efficiency than F2A, E2A, or T2A in human cell lines, zebrafish, mice, and 18 *Drosophila* (10, 14). Often, 2A peptides are used in mammalian cells such as CHO cells 19 (15–17) and human embryonic kidney 293 cells (12) for IgG production. In CHO cells 20 and mice, the use of a 2A self-processing sequence resulted in a higher level of protein 21 productivity compared with that gained by the use of an IRES (12, 17). Whereas some 22 papers have reported recombinant protein production using 2A peptides in insect cells, 23 such as Sf9 cells (18), cells derived from Bombyx mori (18, 19), and Drosophila S2 cells 24 (14), there have been no reports on antibody production utilizing a 2A peptide in insect 25 cells.

1	In the present study, we constructed bicistronic plasmid vectors in which the Hc and
2	Lc genes of an Fab fragment were linked with a P2A self-cleavage peptide sequence, and
3	we investigated the production of Fab fragments in lepidopteran insect cells transfected
4	with a single plasmid. The use of a glycine-serine-glycine (GSG) linker and a furin
5	cleavage site between an Hc and a 2A peptide was also examined. A GSG linker motif
6	is known to improve the self-cleavage efficiency of the 2A peptide (18, 20) by affording
7	greater flexibility (21). Furin is a ubiquitous endonuclease localized in the Golgi
8	apparatus and is highly conserved among eukaryotic species (22, 23). A furin cleavage
9	site is a sequence that is recognized by the proteinase, and it has been added between an
10	Hc and a 2A peptide to remove the 2A residue that remains upstream of the C-terminus
11	of the 2A (12, 16, 24). Furthermore, we also referred to the arrangements of a 2A peptide,
12	a GSG linker, a furin cleavage site, an Hc, and an Lc for efficient production of Fab
13	fragments in insect cells.
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15	MATERIALS AND METHODS
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17	Insect cells and the culture medium Trichoplusia ni BTI-TN-5B1-4 (High
18	Five; Thermo Fisher Scientific, Waltham, MA, USA) cells were cultured in a serum-free

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medium Express Five SFM (Thermo Fisher Scientific) supplemented with 2.6 g/L L-

glutamine (Nacalai Tesque, Kyoto, Japan) and 10 mg/L gentamycin sulfate (Thermo

Fisher Scientific). The cells were maintained at 27°C in T-flasks in a non-humidified

incubator (25). Cell density was determined by microscopically counting the number of

cells using the Countess II automated cell counter (Thermo Fisher Scientific), while cell

viability was judged by the exclusion of trypan blue dye.

1 **Construction of 2A-mediated bicistronic vectors** For the expression of an Fab 2 fragment from a single open reading frame driven by one promoter, the Hc and Lc genes 3 of the 3A21 mouse anti-RNase A (26) Fab fragment were joined by the DNA sequence 4 coding for the 2A peptide from the porcine teschovirus-1 (18). The resultant DNA 5 fragments were cloned into the plasmid vector pIHAneo (3), which contained the B. mori 6 nucleopolyhedrovirus (BmNPV) IE-1 transactivator, the BmNPV HR3 enhancer, and the 7 B. mori actin promoter for high-level expression in insect cells (3, 27). The Drosophila 8 immunoglobulin heavy chain binding protein (BiP) signal sequence (3) was employed 9 upstream of the Hc and Lc genes, respectively (Fig. 1). DNA fragments encoding a GSG \leftarrow Fig. 1 10 linker and a furin recognition site were inserted upstream of the 2A peptide sequence to 11 improve 2A cleavage efficiency and to remove residues of the 2A peptide left after 2A 12 cleavage, respectively, as shown in Fig. 1. The amino acid sequences of the 2A peptide, 13 the GSG linker, and the furin recognition site are shown in Table 1. All the DNA \leftarrow Table 1 14 sequences were codon-optimized for lepidopteran insect cells.

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16 Transient expression and cell culture Transfection grade linear
17 polyethylenimine hydrochloride (PEI) (PEI MAX; Mw 40,000; Polysciences, Warrington,
18 PA, USA) was used as a transfection agent. PEI was prepared at a concentration of 1
19 g/L in 150 mM NaCl, pH 7.0 and sterilized by filtration through a 0.22 μm membrane
20 filter.

In a static culture, High Five cells in the exponential growth phase were inoculated with fresh medium into 6-well plates at a density of 2×10^5 cells/cm³ an hour before transfection. For each transfection, 1 µg of plasmid DNA and 2 µg of PEI per 10⁵ cells were mixed in 150 mM NaCl and incubated at room temperature for 5 min. After incubation, the complex of DNA and PEI was added to the cells, and the cells were then

statically incubated at 27°C. As a positive control, High Five cells were co-transfected 1 2 with a total of 1 µg of pIHAneo separately containing the Hc or Lc gene (5) at a ratio 3 (w/w) of 1:1 (Fig. 1 a). After 72 h, culture supernatants were separated from the cell 4 suspensions via centrifugation and analyzed for the production of Fab fragments. То 5 determine transfection efficiency, the plasmid pXINSECT-EGFP carrying the enhanced 6 green fluorescent protein (EGFP) gene was co-transfected as previously described (5). 7 At 72 h after co-transfection, the cell suspension was removed, and the numbers of green 8 fluorescent cells and total cells were determined using a flow cytometer (Guava easyCyte 9 5HT, Merck Millipore, Darmstadt, Germany).

10 In a shake flask culture, cells in the exponential growth phase were suspended at a 11 density of 2×10^5 cells/cm³ in fresh medium. Fifteen ml of the cell suspension was transferred into a 100-ml screw-capped Erlenmeyer flask. For each transfection, 0.5 µg 12 of plasmid DNA and 1 μ g of PEI per 10⁵ cells were mixed in 150 mM NaCl and incubated 13 14 at room temperature for 5 min. The DNA and PEI complex was added to the cells, and 15 the cells were then incubated at 27°C on a rotary shaker (90 rpm). As a positive control, 16 High Five cells were co-transfected with a total of 0.5 µg of pIHAneo individually 17 carrying either the Hc or Lc gene at a ratio (w/w) of 1:1 (Fig. 1 a). At the appropriate 18 time, 500 μ l of the cell suspension was sampled to measure the cell density, and the 19 culture supernatants were analyzed for the production of Fab fragments.

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21 Western blot analysis Culture supernatants were subjected to sodium dodecyl 22 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% gel under reduced 23 and non-reduced conditions. After electrophoresis, proteins were transferred onto a 24 polyvinylidene difluoride (PVDF) membrane using the iBlot 2 dry blotting system 25 (Thermo Fisher Scientific). Immunoreactive proteins were detected using alkaline phosphatase-conjugated anti-mouse IgG (H + L) (Promega, Madison, WI, USA), which
 is polyclonal antibody that binds to both Hc and Lc of mouse IgG, and staining was
 initiated using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium
 (Promega).

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6 Enzyme-linked immunosorbent assay (ELISA) Culture supernatants were also 7 analyzed by ELISA to evaluate the concentration of Fab fragments with antigen-binding 8 activity as previously described (5). In brief, ELISA plates were coated with bovine 9 RNaseA as the antigen, and horseradish peroxidase-conjugated anti-mouse IgG (H + L)10 (Promega) was used. The ELISA POD substrate TMB kit (Nacalai Tesque) was used 11 for detection according to the manufacturer's protocol. The absorbance was measured 12 with a microplate reader (EnSpire; PerkinElmer Japan, Yokohama, Japan) using a test 13 wavelength of 450 nm and a reference wavelength of 650 nm. The difference between 14 the two absorbances was converted to an Fab fragment concentration by interpolating the 15 value on a predetermined standard curve. The anti-RNaseA Fab fragments were 16 purified from the culture supernatant with NHS-activated Sepharose 4 (GE Healthcare, 17 Little Chalfont, UK) coupled with bovine RNaseA, and were used as a standard.

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19 Statistical analysis Statistically significant differences among data sets were
20 determined using Student's *t*-test. A *p*-value < 0.05 was considered as statistically
21 significant.

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25 Production of Fab fragments using 2A peptide

To examine 2A peptide-

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RESULTS

1 mediated antibody production in insect cells, we constructed a bicistronic expression 2 vector carrying the Hc and Lc genes of the 3A21 Fab fragment linked with a 2A self-3 cleaving peptide sequence from the porcine teschovirus-1 (18). High Five cells were 4 either transfected with a single bicistronic vector (Fig. 1 b) or were co-transfected with a 5 set of plasmid vectors individually containing either the Hc or Lc gene (Fig. 1 a) in 6-6 At 72 h after transfection, culture supernatants were collected, and the well plates. 7 production of Fab fragments was investigated by western blotting under both non-reduced 8 and reduced conditions. Under non-reduced conditions (Fig. 2A), specific protein bands 🗲 Fig. 2 9 were detected at electrophoretic mobilities of approximately 50 and 25 kDa in the culture 10 supernatant of the cells transfected with the bicistronic vector (Hc-2A-Lc) as well as the 11 cells co-transfected with the set of plasmid vectors (Hc & Lc). The electrophoretic 12 mobility coincided with the molecular weight of the 3A21 Fab fragment and that of the 13 Lc monomer, respectively. It should be noted that High Five cells as well as mammalian cells do not secrete Hc alone (5). Under reduced conditions (Fig. 2B), specific protein 14 15 bands corresponding to Hc and Lc monomers were obtained at approximately 25 kDa in 16 the culture supernatant of the cells transfected with the bicistronic vector (Hc-2A-Lc) and 17 of the cells co-transfected with the Hc and Lc genes (Hc & Lc). These results indicate 18 that High Five cells transfected with the bicistronic vector using the 2A sequence 19 successfully secreted Fab fragments in the culture supernatant after self-cleavage of the 20 2A peptide occurred in the cells. However, a low-intensity protein band corresponding 21 to uncleaved Hc-2A-Lc was observed at approximately 50 kDa in the culture supernatant 22 of the cells transfected with the bicistronic vector (Fig. 2B).

23 The effects of a GSG linker and a furin cleavage site were then investigated. When
24 a GSG linker was added to the N-terminus of the 2A peptide (Fig. 1 c), the approximately
25 50 kDa uncleaved protein band disappeared on the western blots under reduced conditions

(Fig. 2B, Hc-GSG-2A-Lc), demonstrating that the GSG linker enhanced the 2A cleavage efficiency. When a furin cleavage site was added to the N-terminus of the 2A peptide (Fig. 1 d), under non-reduced conditions the approximately 50 kDa band corresponding to the Fab fragment shifted to a smaller molecular weight (Fig. 2A; Hc-furin-2A-Lc), which indicates that cleavage successfully occurred at the furin recognition site, and then the residual 2A peptide was removed from the Hc. Hence, the furin cleavage site is useful for the production of Fab fragments without 2A residue in insect cells.

8 The effects of the 2A peptide, the GSG linker, and the furin cleavage site on Fab 9 fragment production were also examined by ELISA of the culture supernatants. The 10 production of Fab fragments with antigen-binding activity was enhanced by adding the 11 GSG linker to the N-terminus of the 2A peptide (Hc-GSG-2A-Lc) as compared with Hc-12 2A-Lc (Fig. 3; p = 0.032). Fab fragment production was depressed by adding the furin \leftarrow Fig. 3 13 cleavage site alone upstream of the 2A peptide (Hc-furin-2A-Lc) compared with Hc-2A-14 Lc. However, the production was improved by using the furin cleavage site along with the GSG linker (Fig. 3; Hc-furin-GSG-2A-Lc vs. Hc-furin-2A-Lc (p = 0.012); Hc-furin-15 GSG-2A-Lc vs. Hc-GSG-2A-Lc (p = 0.058)). The rank order of the GSG linker and the 16 17 furin cleavage site at the N-terminus of the 2A peptide was important, because the plasmid 18 vector Hc-furin-GSG-2A-Lc resulted in productivity that was higher than that with the 19 vector Hc-GSG-furin-2A-Lc (Fig. 3; p = 0.0087). Finally, transfection with the 20 bicistronic expression vector that contained sequences for GSG, the furin cleavage site, 21 GSG, and the 2A peptide between the Hc and Lc genes (Hc-GSG-furin-GSG-2A-Lc) 22 exhibited the highest productivity at a roughly two-fold higher level than conventional 23 co-transfection with a set of plasmids separately containing either the Hc or Lc gene (Hc 24 & Lc) (Fig. 3; p = 0.00084). The transfection efficiency was determined as the 25 percentage of EGFP-positive cells to total cells by flow cytometry at 72 h after

transfection as previously described (5). The transfection efficiency with the bicistronic
 vector Hc-GSG-furin-GSG-2A-Lc was almost the same as that obtained by co transfection of the Hc and Lc genes (data not shown).

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5 Effect of arranging the Lc gene as the first cistron Translation efficiency of 6 the first cistron placed before an IRES was much higher than that of the second cistron 7 located after the IRES (11, 16). Ho et al. (16) showed that arrangement of the Lc gene 8 as the first cistron also returned a higher level of IgG expression compared with use of 9 the Hc gene as the first cistron in 2A-mediated vectors. Higher Lc/Hc gene ratios led to 10 higher levels of Fab fragment secretion in co-transfection of the Lc and Hc genes (5, 16). 11 Taken together, higher productivity of Fab fragments might be expected when the Lc is 12 arranged at the N-terminus of the 2A peptide (Fig. 1 h, i). In the present study, when the 13 plasmid vectors Lc-GSG-2A-Hc and Lc-furin-GSG-2A-Hc were transfected with High 14 Five cells, lower amounts of Fab fragments were detected in both western blotting and 15 ELISA than from the plasmids Hc-GSG-2A-Lc and Hc-furin-GSG-2A-Lc, respectively 16 (Figs. 2C and 3). Interestingly, in western blot analysis under reduced conditions (Fig. 17 2D), approximately 50 kDa uncleaved protein bands were slightly detected.

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19 **Cleavage at the furin recognition site without 2A** To examine the cleavage 20 efficiency of Hc and Lc at the furin recognition site without 2A peptide, the plasmid 21 vectors Hc-furin-Lc and Hc-furin-GSG-Lc (Fig. 1 j, k) were transfected with High Five 22 cells for Fab fragment production. In western blot analysis under non-reduced 23 conditions (Fig. 2E), protein bands corresponding to the Fab fragment were obtained with 24 the plasmid vectors Hc-furin-Lc and Hc-furin-GSG-Lc as well as with Hc-furin-GSG-25 2A-Lc, but an Lc monomer was hardly visible at approximately 25 kDa with both Hcfurin-Lc and Hc-furin-GSG-Lc. Under reduced conditions (Fig. 2F), only uncleaved protein bands were detected at approximately 50 kDa and neither Lc nor Hc monomers were observed with Hc-furin-Lc and Hc-furin-GSG-Lc plasmid vectors. Furthermore, in ELISA (Fig. 3), Fab fragments with antigen-binding activity were scarcely detectable with Hc-furin-Lc and Hc-furin-GSG-Lc. These results confirm that the furin cleavage site alone is insufficient to cleave Hc and Lc and produce Fab fragments with antigenbinding activity.

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9 Production of Fab fragments in a shake-flask culture Since the plasmid 10 vectors Hc-furin-GSG-2A-Lc and Hc-GSG-furin-GSG-2A-Lc gave the highest 11 productivity of Fab fragments with antigen-binding activity in a static culture (Fig. 3), 12 these vectors were individually transfected into High Five cells for Fab fragment 13 production in a shake-flask culture. Cells transfected with bicistronic expression vectors 14 grew in a manner similar to the cells co-transfected with the Hc and Lc genes (Fig. 4A). Fig. 4 15 However, transfection with Hc-furin-GSG-2A-Lc and Hc-GSG-furin-GSG-2A-Lc 16 plasmid vectors exhibited a somewhat higher concentration of Fab fragments than co-17 transfection (Hc & Lc) (Fig. 4B). In the shake culture, the difference in the productivity 18 of Fab fragments was not as significant as in the static culture. The reason for this is not 19 clear, but the difference in the productivity in a shake-flask culture might increase when 20 transfection conditions are optimized. Nevertheless, the plasmid Hc-GSG-furin-GSG-21 2A-Lc gave the highest productivity of Fab fragments in both static and shake-flask 22 cultures.

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DISCUSSION

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1 Production of recombinant antibody molecules such as an Fab fragment as well as an 2 IgG requires a simultaneous expression of two genes encoding both Hc and Lc in an 3 identical cell. In the present study, we constructed plasmid vectors using a 2A self-4 cleaving peptide sequence from the porcine teschovirus-1 with various combinations of 5 GSG linkers and a furin recognition site for bicistronic expression of the Hc and Lc genes 6 of an Fab fragment (Fig. 1). Western blot analysis (Fig. 2) and ELISA (Fig. 3) of culture 7 supernatants showed that High Five cells transfected with the Hc and Lc genes linked 8 with the 2A peptide sequence successfully secreted Fab fragments with antigen-binding 9 activity following self-cleavage of the 2A peptide. The concentration of Fab fragments 10 with antigen-binding activity secreted from the cells transfected with the plasmid vector 11 using the 2A sequence alone (Hc-2A-Lc) was lower than that from the cells co-transfected 12 with the Hc and Lc genes (Hc & Lc), but Fab fragment production was elevated by means 13 of adding a GSG linker and a furin cleavage site at the N-terminus of the 2A peptide (Fig. 14 3).

15 The GSG linker enhanced 2A cleavage efficiency, because in western blot analysis 16 under reduced conditions (Fig. 2B) approximately 50 kDa uncleaved protein bands 17 disappeared by adding GSG to the N-terminus of the 2A peptide when Hc-GSG-2A-Lc 18 and Hc-furin-GSG-2A-Lc were compared with Hc-2A-Lc and Hc-furin-2A-Lc, 19 respectively. In addition, the production of Fab fragments with antigen-binding activity 20 was dramatically enhanced approximately 2-fold by using the GSG spacer at the N-21 terminus of the 2A peptide, when comparing Hc-GSG-2A-Lc, Hc-furin-GSG-2A-Lc, and 22 Hc-GSG-furin-GSG-2A-Lc with Hc-2A-Lc (p = 0.032), Hc-furin-2A-Lc (p = 0.0012), 23 and Hc-GSG-furin-2A-Lc (p = 0.0024), respectively (Fig. 3). Reportedly, the 24 mechanism of a GSG linker could involve reduction in the inhibition rate of the 2A 25 reaction via the tertiary structure of the C-terminal region of the first protein upstream of 1 the 2A peptide (18, 21, 28).

2 The furin recognition site was useful for the removal of the 2A residues from the Hc 3 because a comparison of Hc-furin-2A-Lc with Hc-2A-Lc in western blotting under non-4 reduced conditions (Fig. 2A) revealed that the approximately 50 kDa band corresponding 5 to the Fab fragment was shifted to a smaller molecular weight by adding the furin 6 cleavage site to the N-terminus of the 2A peptide. However, the arrangement of the furin 7 cleavage site directly in front of the 2A sequence (Hc-furin-2A-Lc, Hc-GSG-furin-2A-8 Lc) produced a lower yield of Fab fragments than the use of the GSG sequence between 9 the furin cleavage site and the 2A sequence (Hc-furin-GSG-2A-Lc, Hc-GSG-furin-GSG-10 2A-Lc), respectively (Fig. 3). Interestingly, under reduced conditions a specific band of 11 approximately 28 kDa, probably corresponding to a furin-2A-Lc fusion protein, was 12 detected with both Hc-furin-2A-Lc and Hc-GSG-furin-2A-Lc plasmid vectors. 13 Reportedly, a GSG linker arranged between a furin recognition site and a 2A peptide could 14 enhance the cleavage efficiency at both the furin and 2A cleavage sites by creating more 15 favorable conformations via increased exposure of the furin recognition site at the protein 16 surface and by preventing the inhibition of 2A cleavage caused by interference from the 17 first protein upstream of the 2A peptide (18, 21). These previous studies have shown 18 that a furin cleavage site should keep a proper distance from a 2A sequence in order to 19 achieve efficient cleavage. Chng et al. also reported that only 2A from the porcine 20 teschovirus-1 gave a remarkable difference in Fab fragment expression levels between 21 the absence and presence of GSG between the furin cleavage site and the N-terminus of 22 the 2A in CHO cells (21). Hence, when other 2A peptides (F2A, E2A, or T2A) were 23 adopted in High Five cells, Fab fragment productivity might not be influenced by GSG 24 linkers.

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Reportedly, the order of Hc and Lc genes around a 2A sequence affected the 2A

1 cleavage efficiency and IgG productivity in CHO cells (16). Ho et al. showed that the 2 arrangement of the Lc gene as the first cistron gave a higher level of IgG productivity 3 than that of the Hc gene as the first cistron in both IRES- and 2A-mediated expressions 4 in CHO cells (16). By contrast, in the present study, the arrangement of the Hc gene as 5 the first cistron returned a higher level of Fab productivity (Figs. 2C, D and 3). It is 6 unclear how the arrangement of either the Hc or Lc gene as the first cistron caused 7 differences in the production of Fab fragments, but this might show a correlation with the 8 differences between Hc genes in IgG and Fab fragments.

9 Linking the Hc and Lc genes with only the furin cleavage site without a 2A sequence 10 produced mainly uncleaved Hc-furin-Lc or Hc-furin-GSG-Lc fusion proteins (Fig. 2F), 11 and thus the production of Fab fragments with antigen-binding activity was hardly 12 detectable (Fig. 3). These results agree with a recent study by Lin et al., which showed 13 that linking Hc and Lc genes by a furin cleavage site alone resulted mainly in the 14 production of Hc-furin-Lc fusion protein and a low amount of IgG in CHO cells (29). 15 These results show that the furin cleavage site alone is insufficient to cleave Hc and Lc 16 expressed from a single open reading frame.

17 In the present study, GSG-furin-GSG-2A-mediated expression of the Hc and Lc genes 18 (Hc-GSG-furin-GSG-2A-Lc) exhibited the highest productivity of Fab fragments via 19 either a static (Fig. 3) or a shake-flask culture (Fig. 4B) of transfected High Five cells. 20 These results demonstrate that bicistronic expression using the appropriate arrangement 21 of GSG linkers, a furin recognition site, and a 2A peptide allows the efficient production 22 of antibody molecules consisting of multiple polypeptides in insect cells. The 23 productivity in a shake-flask culture could be further improved by optimization of 24 transfection conditions. More detailed analyses of Hc and Lc polypeptides after 2A 25 cleavage, such as amino acid sequence determination, have not yet been performed. A

1	comparative study using different 2A peptides is also necessary in order to select the	
2	optimal 2A peptide in insect cells.	
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1 Figure captions

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3 FIG. 1. Schematic representation of bicistronic vectors for Fab fragment production in 4 insect cells. Each fragment was ligated with pIHAneo vector. Drosophila BiP signal 5 sequence (striped box) was used upstream of every heavy chain (Hc) and light chain (Lc) 6 gene of the 3A21 Fab fragment. As a positive control, the Hc or Lc gene of the Fab 7 fragment was respectively ligated to pIHAneo (a), and High Five cells were co-8 transfected with both plasmid vectors. Only construct (a) contained both a V5 epitope 9 tag sequence and a His tag sequence at the 3' end of the Lc gene (dotted box). Actin, 10 Bombyx mori cytoplasmic actin promoter; 2A, DNA encoding a 2A peptide derived from 11 the porcine teschovirus-1; G, DNA encoding a GSG linker; F, DNA encoding a furin 12 recognition site; pA, OpIE2 polyadenylation sequence from the Orgyia pseudotsugata 13 nucelopolyhedrovirus.

14

FIG. 2. Western blot analysis of culture supernatant of transfected cells under nonreduced (A, C, E) and reduced conditions (B, D, F). (A, B) Bicistronic vectors containing the Hc gene upstream of the 2A sequence and the Lc gene downstream of the 2A sequence (Fig. 1 a–g) were transfected with High Five cells in 6-well plates. Control, untransfected cells. (C, D) Bicistronic vectors containing the Lc gene as the first cistron in front of the Hc gene (Fig. 1 h, i) were transfected. (E, F) Bicistronic vectors without the 2A sequence (Fig. 1 j, k) were used.

22

FIG. 3. Enzyme-linked immunosorbent assay (ELISA) of the cell-culture supernatant.
Bars represent the means with error bars indicating S.E. obtained from three independent
experiments in static cultures. Control, untransfected cells. Asterisk indicates

- 1 significant difference (* p < 0.05; ** p < 0.01).

FIG. 4. Time course of viable cell density (A) and concentration of Fab fragments in
the culture supernatant (B) in shake-flask cultures of transfected High Five cells. Bars
represent the means with error bars indicating S.E. obtained from three independent
experiments.

TABLE 1. List of amino acid sequences

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Name	Sequence
P2A	ATNFSLLKQAGDVEENPGP
Furin cleavage site	RRKR
GSG linker	GSG
Нс	MDVQLQESGPGLVKPSQSLSLTCTVTGYSITSDYAWNWI
	RQFPGNKLEWMGYISHSGSTGYNPSLKSRISITRDTSKN
	QFFLQLNSVTTEDTATYYCARGGKNWDAYWGQGTLVT
	VSSKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPV
	TVTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVPSSTWPS
	ETVTCNVAHPASSTKVDKKIPRDCGAAALE
Lc	MDIKMTQSPSSMYAFLGERVTITCKASQDINSYLSWFQQ
	KPGKSPKTLIYRANRLVDGVPSRFSGSGSGQDYSLTISSL
	EYEDMGIYYCLQYDELPFTFGSGTKLEIKRADAAPTVSI
	FPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQ
	NGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTC
	EATHKTSTSPIVKSFNRNECAAALE
BiP signal	MKLCILLAVVAFVGLSLG









